AFRL-ML-TY-TP-2007-4544

PREPRINT

SILICA-IMMOBILIZED ENZYME REACTORS

Heather R. Luckarift
Air Force Research Laboratory

August 2007

Submitted for publication in the Journal of Liquid Chromatography and Related Technologies.

Air Force Research Laboratory
Materials and Manufacturing Directorate
Airbase Technologies Division
139 Barnes Drive, Suite 2
Tyndall AFB, FL 32403-5323

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
10-SEP-2007	Journal Article PREPRINT	06-NOV-2006 - 30-AUG-2007

4. TITLE AND SUBTITLE

Silica-Immobilized Enzyme Reactors

5a. CONTRACT NUMBER

FA4819-07-D-0001

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

62102F

6. AUTHOR(S)

Luckarift, Heather R.

5d. PROJECT NUMBER

4915

5e. TASK NUMBER

L2

5f. WORK UNIT NUMBER

Q140LA62

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Air Force Research Laboratory
Materials and Manufacturing Directorate
139 Barnes Drive, Suite 2
Tyndall Air Force Base, FL 32403-5323

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Research Laboratory
Materials and Manufacturing Directorate
139 Barnes Drive, Suite 2
Tyndall Air Force Base, FL 32403-5323

10. SPONSOR/MONITOR'S ACRONYM(S)

AFRL/MLQL

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

AFRL-ML-TY-TP-2007-4544

12. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution Statement A: Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

Ref AFRL/MLQ Public Affairs Case Number 07-063. Submitted for publication in the Journal of Liquid Chromatography and Related Technologies. Document contains color images.

14. ABSTRACT

Recent studies have demonstrated the applicability and versatility of immobilized enzyme reactors (IMERS) for chemical and biochemical synthesis and analysis. The majority of IMER systems rely on enzymes immobilized to packed matrices within flow-through devices. This review focuses primarily on the use of silica as a support for enzyme immobilization and specific applications of the resulting silica-based IMERs. A number of recently published examples (from 2000 onwards) are discussed as model systems. The effect of various silica matrices and immobilization techniques upon the enzymatic properties and stability of the biocatalysts is considered. In particular, reports in which the carrier matrix is biologically-derived silica is emphasized and demonstrated as a versatile technique in terms of advantageous recovery, reuse and reproducibility.

15. SUBJECT TERMS

Immobilized enzymes, enzyme reactors, silica-immobilization, biocatalysis, drug discovery, biosilicification

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Glenn Johnson
U	U	U	UU	38	19b. TELEPHONE NUMBER *(Include area code)*

Reset

Silica-Immobilized Enzyme Reactors

Heather R. Luckarift

Microbiology and Applied Biochemistry, Air Force Research Laboratory, 139 Barnes Drive, Suite # 2, Tyndall AFB, FL 32403

Tel: 850-283-6034, Fax: 850-283-6509, Email: hluckarift@gtcom.net

Abstract

Recent studies have demonstrated the applicability and versatility of immobilized enzyme reactors (IMERs) for chemical and biochemical synthesis and analysis. The majority of IMER systems rely on enzymes immobilized to packed matrices within flow-through devices. This review focuses primarily on the use of silica as a support for enzyme immobilization and specific applications of the resulting silica-based IMERs. A number of recently published examples (2000 onwards) are discussed as model systems. The effect of various silica matrices and immobilization techniques upon the enzymatic properties and stability of the biocatalysts is considered. In addition, reports in which the carrier matrix is biologically-derived silica are highlighted as an alternative and versatile technique that provides advantageous recovery, reuse and reproducibility.

Keywords: Immobilized enzymes, Enzyme reactors, IMER, Biocatalysis, Drug discovery, Biosilica.

Introduction and Scope

Enzymes are versatile catalysts that mediate a diversity of metabolic and pharmacological processes. Significant advancements in genetic engineering and protein purification methods have resulted in a wealth of commercially available enzymes which are readily isolated from growing culture. Although the diversity of available enzymes has expanded, issues related to their integration and stability during chemical production and analysis still exist. In their native state, enzymes are soluble in aqueous solutions and as such, are often susceptible to denaturation once separated from their physiological environment. By their nature, enzymes are not consumed during the reactions that they catalyze, but are difficult to reuse and recover as a soluble component from a reaction mixture. This is a primary concern for enzymes which are expensive to prepare and only available in very small amounts. Immobilization of enzymes is therefore often used in an attempt to stabilize and prolong the activity and reusability of the catalyst. Numerous methods for enzyme immobilization have been explored and offer a variety of advantages depending upon the functional and biochemical properties of the enzyme, as well as the final application.[1-5]

The integration of immobilized enzymes into continuous flow-through systems provides an added advantage to many applications, as the immobilized biocatalyst is stabilized and can be recycled. The resulting immobilized enzyme reactors (IMERs) can also be integrated directly to further analytical methods such as liquid chromatography or mass spectrometry.[6] In fact, one of the primary applications of many IMER systems is the digestion of proteins for automated sequence analysis by integration with mass spectrometry. The use of IMERs for protein digestion, however, has been reviewed extensively elsewhere and will not be covered herein.[6-10]

A variety of matrices have been investigated over the years for enzyme immobilization or encapsulation, but for focus, the emphasis within this review is limited to IMERs that utilize

silica as a scaffold. The use of silica-based IMERs has received increasing attention since their appearance in the early 1970's and since then, the use and diversity of applications has grown steadily (Figure 1). The last decade has seen a vast increase in this subject area; presumably due to a parallel development of commercially available chromatographic support matrices that are amenable to IMER development. IMERs now find application in scientific fields as diverse as pharmacology, chemistry, materials science and chemical engineering (Figure 1, inset). With a few exceptions that are included for reference, the work reviewed herein will be restricted to studies reported since the year 2000.

Enzyme immobilization

Enzyme immobilization is generally achieved through covalent binding, ionic or hydrophobic interactions, or physical entrapment. Covalent attachment is one of the most commonly used methods for stabilizing enzyme activity as the enzyme molecule becomes fixed in a manner that limits the conformational changes that can lead to enzyme denaturation.[11-13] There are intrinsic problems, however, in that covalent attachment can lead to limitations and variations in conformational mobility due to the orientation of the enzyme on the support during immobilization. Immobilization mediated by ionic or hydrophobic interactions, by comparison, is much less rigid but the adsorbed enzyme may leach from its support upon slight changes in the reaction environment (e.g. pH, ionic strength or temperature). Overall, however, immobilization using physical interactions provides an environment that greatly improves the catalytic properties of the biocatalyst, particularly in non-physiological environments.[14-16] Sol-gel encapsulation has also been widely studied for enzyme immobilization, but the primary drawbacks are poor loading efficiency and enzyme leakage.[17,18] The limitation has been resolved for some enzymes by

fabrication of support matrices with pore sizes that are specifically tailored to allow substrate flow but prevent enzyme leaching.[19]

Biosilicification is the formation of nano-patterned siliceous structures in biological systems and is mediated by proteins such as silaffins and silicateins, found in marine diatoms and sponges, respectively.[20-22] Using biological molecules as scaffold templates, biomimetic silicification reactions have been shown to provide a simple and effective alternative for enzyme encapsulation.[23,24] The biologically-derived silica particles physically entrap enzymes within silica nanospheres directly as the silica particles form, providing an effective 'cage' that limits enzyme denaturation and retains high enzyme activity. The application of biologically-derived silica (biosilica) as a method for enzyme immobilization and preparation of IMERs using this method will be discussed in more detail below.

Immobilized enzyme reactors

The packaging of immobilized enzymes within a flow-through system creates an IMER that can be utilized as a microreactor (for chemical synthesis) or integrated into conventional chromatography systems (for analyte detection).[25,26] IMERs can be exploited either before (pre-column) or after (post-column) a chromatographic separation or can serve as a chromatographic column in itself. Pre-column conversions would include pre-treatment of analytes such as chiral separation or screening for enzyme inhibition. Location of an IMER before a chromatography column via a switching valve allows the IMER to be separated from denaturing conditions downstream (i.e. organic solvents or pH environments required for subsequent chromatographic separations).[27,28] Post-column IMERs are primarily designed to enhance detection of a product or analyte.[29-32] As the diversity of support matrices escalates, the difficulty in defining optimum

characteristics relevant to all applications becomes more difficult. The choice of support is dependent upon the nature and mechanism of the enzyme, the properties of the support and the final application and conditions of use of the resulting IMER. A review of pre and post-column IMER formulations, support matrices and their relative advantages and disadvantages is provided by Girelli *et al*.[33]

IMERs are useful for the analysis of biological systems only as long as the characteristics of the biomolecule are retained. The ability to study enzyme kinetic characteristics using IMER systems by calculation of Michaelis-Menten Kinetics has been demonstrated to confirm that the immobilized enzyme is not significantly hindered by its orientation and binding.[8,34] Lineweaver-Burk plots of reaction rate *vs.* substrate concentration are readily achievable in flow-through IMER systems from which the Michaelis constant (K_m) and the maximum enzyme velocity (V_{max}) can be calculated and mechanisms of inhibition (competitive, noncompetitive etc.) can be determined. Specific enzyme inhibition affinity can also be calculated in IMER systems by measuring the IC_{50} (the inhibitor concentration that results in a 50% reduction in product conversion under saturating substrate conditions) or an inhibition constant (K_i) calculated from the intersect of a Lineweaver-Burk plot.[35,36]

Silica as an enzyme-immobilization support

Various support matrices have been developed and are now commercially available for enzyme immobilization. Of all available techniques, silica-based derivatized matrices, monolithic chromatography supports and artificial membrane stationary phases are the most commonly used. Using these techniques, the immobilization of enzymes to various stationary phases has now been demonstrated for a wide variety of applications using a range of enzymes, membrane proteins and receptor proteins.[19,37-41] Silica-based stationary phases of the type used

in liquid chromatography separation columns are commonly used as support matrices for enzyme immobilization to produce IMERs. The primary advantage of the readily available commercial supports is their reproducibility. The silica support, however, must be functionalized to allow for covalent attachment of enzymes. An additional limitation of these columns is that the void volume of packed silica columns may create diffusion and flow limitations. The recent introduction of monolithic polymers as a stationary phase provides an alternative to overcome those limitations.[10,19,42-45] Monolithic separation media is essentially one single core which does not contain voids between the packing materials. This results in improved mass transfer, as all of the sample and mobile phase must flow through the entire stationary phase. Silica-based monoliths provide an inherent advantage of having equally sized mesoporous structures and large surface areas. The high-throughput of monoliths allow for high flow rates with low back pressures; which enables further coupling to analytical detection systems. Selection of silica monoliths, however, is often a trade off between macroporous structures that permit high flow rates and low back pressures but with significant leaching of protein (due to large pore sizes >50 nm), compared to mesoporous structures with pore diameters more suited to protein retention (3-5 nm) but a concurrent increase in high back pressures even at low flow rates. Besanger *et al*., for example, reported a mesoporous silica monolith that generates a back pressure of >3500 psi at flow rates of less than 1 μl/min, which excludes further integration with pressure-driven liquid chromatography.[19] The authors attempted to address the limitation using sol-gel synthesis; using glyceroxysilanes to create mesoporous materials that combines high protein retention of α-glutamyl transpeptidase (loading efficiency >80%) with enhanced flow characteristics. One limitation of this approach was long preparation times of several days, due to multiple aging steps. Variations in fabrication pH and polyethylene glycol concentrations were optimized to minimize shrinkage of the monoliths during preparation and to prevent flow-channeling as a

result of the column pulling away from the capillary surface. Flow rates were low in the final system, with optimal rates less than 1 μl/min. The apparent catalytic activity of the immobilized enzyme, however, was comparable to soluble enzyme for the conversion of L-glutamic acid *p*-nitroanilide to *p*-nitroaniline.[19] The sol-gel method can be used to produce an aggregate of spherical particles that occurs due to phase separation during sol-gel transition, resulting in a coarse porous matrix with good flow characteristics when packed into a flow-through column. The particles formed using these techniques are of the order of several microns in diameter with interparticle gaps of the same size order that allow the efficient flow of substrate through the column. Sol-gel entrapment of protease, for example, has been demonstrated in an IMER for transesterification reactions.[42]

An alternative area of IMER development is the use of immobilized artificial membrane (IAM) stationary phases that consist of a monolayer of phospholipid covalently immobilized on an inert silica support, in which enzymes are entrapped in an environment that resembles a biological membrane. IAM stationary phases are particularly useful for studying trans-membrane receptors and drug transport across membranes and for non-covalent immobilization of membrane-associated proteins. A wealth of reports on the use of IAM stationary phases to study drug interactions, particularly across the blood-brain barrier, are documented but not described further herein due to the depth of the subject area.[46-55]

Biosilica for IMER preparation

Biomimetic silicification reactions provide a rapid and simple alternative method for enzyme immobilization that result in the physical entrapment of enzymes within silica nanospheres as they are formed (Figure 2). The reaction mixture consists of a silica-precipitating

peptide and a silicate precursor (tetramethylorthosilicate) that rapidly (<2 minutes) form silica nanoparticles in aqueous buffered solution. A matrix of spherical silica particles (typically ~500nm in diameter) is formed and provides effective encapsulation of a range of enzymes including esterases, hydrolases, peroxidases and reductases.[23,35,56-59] The silica particles can be further integrated into flow-through systems by either: i) preparing the silica particles in batch mode and then packing them into a column or by ii) preparing the silica particles within a commercially available pre-packed column, *in situ*, by anchoring them to the column matrix. Both alternatives provide viable methods for fabricating IMERs and examples of each are included within this review. To achieve biosilica formation (and enzyme encapsulation) *in situ*, the silica-precipitating peptide is functionalized with a histidine-rich tail (his-tag), allowing for its retention within a commercial pre-packed column by metal affinity binding between the 'his-tag' and a metal coating (cobalt) upon the agarose packing material. The peptide forms silica *in situ* and simultaneously encapsulates enzymes directly at the surface of the agarose and hence within the packed column (Figure 2). To our knowledge, the use of biologically-derived silica matrices (biosilica) as a support matrix for development of IMERs is unique and will be described in more detail throughout this review to highlight the validity of the approach.

IMERs for drug discovery

Many basic biological signaling responses rely upon specific interactions between biomolecules and a respective, and often highly specific, ligand. As such, pharmaceutical research is often directed towards the elucidation of these mechanisms and their regulation or modulation by drug interactions.[60] Although enzyme activation has a therapeutic role, the mode of action of many commercially available drugs is enzyme inhibition. Pharmacological inhibition of cyclooxygenase, for example, can provide relief from the symptoms of inflammation and pain

and is the mode of action of drugs such as aspirin and ibuprofen.[61] Serotonin reuptake inhibitors and monoamine oxidase inhibitors can function as antidepressants, due to their ability to prevent the breakdown of neurotransmitters.[62-64] Screening of therapeutic enzyme inhibitors is often directed towards achieving high specificity and increased potency in order to reduce unspecific interactions (i.e. side effects) and lower toxicity. Examples of IMERs with application to drug discovery and metabolic screening are highlighted in the following sections.

Analysis of cholinesterase inhibition

The pharmacology of cholinesterase enzymes is an area of increasing interest as inhibitors of cholinesterases have therapeutic value for treatment of neurogenerative diseases such as dementia and Down's syndrome.[65-67] In addition, cholinesterase inhibitors are the only currently FDA approved treatment for Alzheimer's disease.[68-70] Cholinesterases such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) catalyze the hydrolysis of acetylcholine; a neurotransmitter in the central nervous system. Cholinesterase inhibitors bind to the enzyme and prevent the enzymatic breakdown of acetylcholine. Acetylcholine accumulates as a result, overwhelming the central nervous system and leading to ataxia (failure of muscle coordination and movements), seizures and ultimately death. In addition, the toxicity of cholinesterase inhibitors, particularly irreversible inhibitors, has led to their use as nerve agents in warfare.[71,72]

A range of IMER preparation methods have been investigated for immobilization of cholinesterase enzymes in order to screen for enzyme inhibitors (Table 1). A comparison of silica-packed columns and monolithic columns for IMER preparation was recently reported by Bartolini *et al*.[73] Two modified monoliths (with epoxy or ethylenediamino reactive groups)

were prepared and compared to a silica-packed column for the immobilization of AChE and kinetic characteristics of the columns were determined at a range of flow rates. The monolithic columns demonstrated specific advantages; good stability, enzyme loading is performed *in situ* and conditioning times are short. The loading capacity of the columns were low (~3%), however, compared to silica-packed columns (~29%). The silica-packed column also required a shorter preparation time and at a fraction of the cost. AChE immobilization was also achieved by *in situ* derivatization of epoxide silica that resulted in covalent linkages between the enzyme and the epoxide groups and provided stability to the immobilized enzyme. The loading capacity of the column was high (Table 1) but analysis times were reduced due to a requirement for low flow rates.[74] By comparison, immobilization of BuChE in biosilica nanoparticles was complete in less than 1 hour with an immobilization efficiency of 100% and a high loading capacity for enzyme (Table 1).[35] Biosilica-IMERs containing immobilized BuChE were used for screening the drug potency of cholinesterase inhibitors (Figure 3) and were stable for over 2 days of continuous use at flow rates of up to 3 ml/min.[35]

In an alternative system, AChE was immobilized to silica gel by covalent binding and used to monitor the hydrolysis of acetylcholine and concurrent inhibition by carbamate pesticides (Table 1). The system was reusable, but only a few times and as such was designed as a disposable sensor for pesticides. AChE hydrolyses acetylcholine to release choline and acetic acid. The acid undergoes spontaneous dissociation and releases hydrogen ions which can be detected potentiometrically (change in pH due to increase of hydrogen ions) or conductimetrically (change in conductivity due to increase in ion concentration). Both detection systems in conjunction with the AChE-IMER provided detection limits of less than 1 ppm and within range for detection of carbamates at environmentally relevant levels.[75]

The interest in cholinesterase enzymes and their reaction metabolites is exemplified by the availability of a commercial polymeric bioreactor available from Bioanalytical Systems Inc. (BASi, West Lafayette, IN) for analysis of acetylcholine and choline. Dong *et al*., reported a modification to the commercially available product, by the addition of horseradish peroxidase in a post-column IMER that improved sensitivity of detection by 200-fold. Horseradish peroxidase was immobilized to amine-functionalized silica beads and packed into a column and used to monitor the formation of hydrogen peroxide as a measure of acetylcholine concentration in mammalian lysates. The IMER column was stable for up to 8 months with no loss in activity, providing that a biocide was added to prevent the growth of contaminating biofilms.[76]

Drug metabolism by glucuronidation

Glucuronidation plays a significant role in the pharmacology of many drugs and as such, the determination of non-conjugated and conjugated drug metabolites is a useful tool. The reaction primarily converts toxins and certain drugs to a more water soluble metabolite that can be readily excreted from the body by the kidneys. Uridine diphospho-glucuronosyltransferase (UDPGT), for example, is involved in metabolic detoxification in vertebrates through glucuronidation reactions that facilitate the transport of lipophilic compounds to excretory organs. Non-solubilized UDPGT from rat liver microsomes was covalently immobilized to a functionalized silica support by Schiff base chemistry and a number of reaction variables (i.e. sonication treatments and buffer selection) were investigated during immobilization to determine an optimum configuration.[77] A high loading capacity for protein (~40 mg protein g^{-1} silica) was a trade off with a reduction in specific activity that was attributed to destruction of lipids associated with the enzyme during preparation. IMER preparation times of up to 3 days may also contribute to a loss in specific activity. The resulting UDPGT-IMERs, however, was reusable

over a period of 45 days with up to 75% activity retained, depending upon the IMER configuration. The IMER was coupled (pre-column) to a C_{18} and anion-exchange column in order to separate substrate from product downstream and used for the on-line glucuronidation of 4-methylumbelliferone and acetaminophen.[77] Immobilization of rat liver microsomes containing UDPGT using silica sol-gel technology to create an IMER for capillary electrochromatography has also been reported and used for the on-line analysis of the metabolism of *p*-nitrophenol and testosterone.[78] Similarly, β-glucuronidase was immobilized to a monolithic silica based column and used to evaluate the conversion of the cough suppressant dextromethorphan to two metabolites; dextrorphan and 3-hydroxymorphinan. The products are formed by cytochrome P-450 enzymes and undergo glucuronidation reactions *in vivo*. As such, the presence of these metabolites in urine is used to assess the metabolic activity of specific cytochrome P-450 enzymes. The resulting silica-based monolithic IMER of β-glucuronidase provides on-line hydrolysis of the conjugates for rapid analysis of metabolite concentrations in human urine samples.[79]

Determination of catecholamines

Catecholamines such as norepinephrine, epinephrine and dopamine are an important group of chemical compounds that act as neurotransmitters in the central nervous system (Figure 4).[80] The work of Markoglou and Wainer has demonstrated the application of IMER systems for the analysis of catecholamines in order to understand the physiological interaction with various adrenergic receptor proteins.[81-85] Dopamine β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) are essential enzymes involved in the metabolism of catecholamines *in vivo*. These enzymes are essential to nervous signaling and

implicated in neurological disorders such as Schizophrenia and Parkinson's disease.[86] Drug discovery for targets that can alter the function of these enzymes are therefore a key focus. The immobilization of PNMT and DBH to glutaraldehyde-functionalized silica gel was optimized in batch systems (~2.5 mg protein g^{-1} support) and packed into columns.[81-83] Immobilization of PNMT had some deleterious effects upon enzyme activity but the system was stable at elevated temperatures (up to 60^{o}C) and proved effective at screening the binding characteristics of known inhibitors. An IMER based on immobilized PNMT, for example, was used to demonstrate the *N*-methylation of normetanephrine (Figure 4, R = OCH_3).[81] In addition, the PNMT-IMER was coupled with a second DBH-IMER for two-step synthesis of L-epinephrine from dopamine, with product recoveries of ~95% (Figure 4). The technology was extended to incorporate two different forms of DBH (soluble and membrane-bound) by utilizing two differing methods of IMER preparation; an immobilized artificial membrane for the membrane-bound protein and glutaraldehyde-functionalized silica for the soluble protein.[85]

Screening for prodrug activation

Berne *et al*. describe an IMER containing a bacterial nitrobenzene nitroreductase enzyme that catalyzes the conversion of a nitro group to hydroxylamine resulting in a large electronic change which can be exploited for a variety of biotechnological applications; primarily the activation of prodrugs and proantibiotics for cancer treatments or antibiotic therapy, respectively.[87] Nitrobenzene nitroreductase was immobilized (>80% immobilization efficiency) in a silica matrix formed by polyethyleneimine (PEI) that catalyzes the formation of a matrix of interconnected silica particles of ~1 μm diameter. PEI has been extensively used for stabilizing enzymes by generating hydrophilic microenvironments that protect the enzyme from

denaturation and the use of PEI as a scaffold for silica formation therefore provides a synergism between the stabilizing effect of PEI and the encapsulation stability of silica nanoparticles. The resulting silica-encapsulated nitroreductase was contained in a stainless steel column to create an IMER for conversion of nitrobenzene, a prodrug (CB1954), and a proantibiotic (nitrofurazone). The IMER showed excellent activity and stability for the screening of prodrugs in non-aqueous solvents such as methanol and acetonitrile. IMER functioning in a non-aqueous system is a primary advantage, due to the relative insolubility of many prodrugs in aqueous solutions. Flow rates within the system were low (1-5 μL/min) but no enzyme leaching or back-pressure problems were reported and stoichiometric conversion of all test substrates was observed, with a concurrent decrease in conversion efficiency with increased flow rates as expected. The system was operated for more than 3 days at room temperature with a flow rate of 5 μL/min for continuous conversion of nitrobenzene with >90% conversion efficiency (Figure 5).

Screening inhibitors of glyceraldehyde-3-phopshate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a target for developing drugs for the treatment of parasitic diseases such as sleeping sickness, due to its role in controlling ATP production in pathogenic parasites.[88] GAPDH, however, has low stability and loses up to half of its activity within a day. GADPH isolated from rabbit was covalently immobilized to a wide-pore silica support by glutaraldehyde activation and Schiff-base chemistry.[89] The loading capacity was 150 μg of enzyme to 100 mg stationary phase with ~95% loading efficiency, but less than 3% of the initial enzyme activity was retained. The low retention of activity was attributed to the instability of the enzyme in aqueous solutions and the significant time delay (6 hours at optimum) for binding to occur. The resulting particles, however, were packed into a

glass column to make a GAPDH-IMER which was stable for 30 days; a significant improvement upon the native enzyme. The IMER was utilized for kinetic measurements of enzyme activities and enzyme inhibition was evaluated with the toxin, agaric acid, to demonstrate that the IMER could be used for screening GAPDH inhibitors.

IMERs for chemical synthesis

Enzymes are useful for synthesis of fine chemicals, production of agrochemicals and pharmaceuticals.[90-96] In order to employ enzymes in bioprocesses a number of limitations such as enzyme stability, difficulty in separating the product from the catalyst and the inherent difficult of re-using the catalyst must be overcome. These disadvantages can be alleviated by using IMER systems that provide enzymes immobilized into, or onto, a solid support. The resulting immobilized catalysts are heterogeneous and can be readily separated and recovered; increasing the commercial feasibility of enzyme-based reactions. Separation of products downstream of the process can raise production costs considerably and in this area, the development of continuous flow reaction systems has seen a critical input to improving technology. IMERs have now been developed by a number of groups and demonstrated as a viable alternative to conventional synthesis. The immobilization of subtilisin Carlsberg to fumed silica, for example, was used within a continuous packed-bed reactor for transesterification reactions in hexane.[97] In the following section, examples of catalysis using IMER systems are chosen to exemplify the application to chemical synthesis.

Preparation of penicillin analogues

Penicillin G acylase (PGA) enzymes catalyze the cleavage of the acyl-chain of penicillins to produce 6-aminopenicillanic acid; providing a commodity product at a scale of 20,000 tons per annum. The enzyme has very broad substrate specificity with utility to the production of semi-synthetic penicillins and β-lactam antibiotics.[98,99] The enzyme can resolve racemic mixtures of chiral compounds with excellent stereochemistry for a range of substrates. The catalytic versatility of the enzyme has been extended by immobilizing PGA onto chromatography supports and using the enantiomeric selectivity of the enzyme to resolve racemic mixtures.[100] Immobilization onto supports with various pore sizes and functionalities were recently compared for the development of a PGA-IMER using two preparation methods: i) immobilizing the enzyme and then packing the immobilized catalyst into a column (batch) versus ii) immobilizing the enzyme within a pre-packed column (*in-situ*). Despite the high loading capacity observed with batch preparation (87 mg enzyme g^{-1} support), a loss of activity was observed upon packing and *in-situ* techniques provided optimal performance in comparison. Immobilization *via* the amino groups of the enzyme showed retention of activity, whereas enzyme activity was abolished when the enzyme was attached via its carboxyl groups.[101] Microparticulate epoxy-silica supports showed high enzyme loading but the efficiency was greatly superseded by single phase porous silica monoliths where ~250 mg of protein could be loaded to a single column. Using the optimized system, PGA-IMERs were integrated with an liquid chromatography system to determine the PGA-catalyzed hydrolysis of esters. In addition, the resulting column acted as a chiral stationary phase for screening of substrate analogues and non-steroidal anti-inflammatory drugs.[100] Further downstream chiral separation led to precise determination of enantioselectivity in the synthesis of 2-aryloxyalkanoic acid methyl esters and a variety of substrate analogues.[102]

Lipase-catalyzed conversions

Lipases are one of the most versatile and hence widely exploited groups of enzymes in biocatalysis.[103-108] In the previous section we noted that PGA can be utilised to synthesize 2-aryloxyalkanoic acid methyl esters. A similar report utilizes a lipase from *Candida rugosa* for racemic separation of 2-aryloxyalkanoic acids, analogous methyl esters and non-steroidal anti-inflammatory drugs.[109] Lipase based IMERs were produced by i) physical adsorption of lipase to a silica (RP18) stationary phase and by ii) covalent immobilization of lipase *via* activating agents on aminopropyl-functionalized silica. Immobilization yield by physical adsorption was high but unstable due to weak hydrophobic interactions which allowed the enzyme to leach from the IMER over time; particularly in the presence of solvent. Covalent immobilization eliminated the leaching problems, allowing for chromatographic separation and collection of products for off-line enantioselective analysis.

Lipase from *Candida rugosa* was immobilized to silica gel with relatively low loading capacity (1.9 mg protein g^{-1} support) but good recovery of activity (~37%) and used as a packed-bed IMER to catalyze the racemic resolution of (S)-ketoprofen from its constituent enantiomers. The optically pure (S)-isomer was obtained with >99% ee at a conversion rate of ~30% and a productivity rate of 1.5 mg g^{-1} biocatalyst h^{-1}. (S)-ketoprofen is a non-steroidal antiflammatory drug used to reduce inflammation and relieve pain; the (R)-isomer has no activity.[110,111] By comparison, 2-phenoxypropionic acids and their esters are used as herbicides; in this case, the (R)-isomers are biologically active. Lipase from *Candida rugosa* was again immobilized to silica beads and used as a packed-bed reactor for the continuous racemic resolution of 2-(4-chlorophenoxyl) propionic acid (46% ee).[112]

Conversion of nitroarenes

One advantage of immobilizing enzymes is the ability to utilize a biocatalyst in non-physiological environmental conditions. The combination of a metal and a biocatalyst, for example, would ordinarily not be feasible due to the disparate reaction conditions required for optimal activity. An IMER containing biosilica-immobilized hydroxylaminobenzene mutase (HABM) was developed using a simple fluidized-bed design and integrated with a packed-bed reactor containing zinc for conversion of nitrobenzene to *o*-aminophenol with ~90% conversion efficiency during continuous operation over a period of 24 hours. The HABM-IMER system also proved amenable to the formation of a novel homologue of the antibiotic, chloramphenicol (Figure 6).[56] The system can operate at flow rates of the order of milliliters per minute (for large scale catalysis) but can be reduced in scale to microfluidic formats with flow rates of only microlitres per hour (more suited to screening). A microfluidic HABM-IMER was used in conjunction with an additional microfluidic IMER connected in series that contained biosilica-immobilized soybean peroxidase for the synthesis of 2-aminophenoxazin-3-one; an intermediate in the synthesis of actinomycin antibiotics (Figure 6). [58]

IMERs as biosensors

The majority of drug interactions rely on reversible inhibitors of enzyme activity. Irreversible enzyme inhibitors, however, can often cause drastic effects upon metabolic functions and are the mechanism of action of many poisons and potent neurotoxins. Ricin, for example, is a potent protein toxin found in castor oil beans. Ricin is an irreversible inhibitor that functions as a glycosidase and inactivates ribosomes so specifically that fewer than ten castor oil beans is sufficient for severe cytotoxicity and potential fatality.[113,114] As such, ricin and other

neurotoxins also find application as terrorist threat agents. One area of research applicable to IMERs has been the development of biosensor systems to screen irreversible inhibitors despite limitations that are inherent to this type of system; such as enzyme reactivation. Although a close correlation between biosensors and IMERs may seem apparent, according to the strict IUPAC definition of a biosensor, an IMER by itself is not a biosensor. A biosensor is defined as 'a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds, usually by electrical, thermal or optical signals'.[115] The integration of an IMER with a detection system may therefore lead to the development of a biosensor but IMERs are essentially bioreactors rather than biosensors as the biological component is distinct from the physical transducer.[114] The distinction, however, is not always evident and examples of biosensors/IMER combinations can be found within the literature. A number of examples of such are included in a review by Fishman et al.[116] The IMER containing biosilica-immobilized BuChE described earlier for screening the drug potency of cholinesterase inhibitors, for example, was also integrated with an aerosol collection system to develop a biosensor for the detection of organophosphate nerve agents in air.[59] The system was tested with model organophosphates including paraoxon, demeton-S and malathion. The substrates are all potent inhibitors of BuChE and as such, the BuChE-IMER required reactivation with pyridine-2-aldoxime following every test sample in order to maintain enzymatic activity over repeated use. Despite the inherent limitations, the system proved suitable for detection of organophosphates in air at practical detection limits.[59]

Conclusions and Future considerations

The application of silica-based IMERs clearly offers practical advantages, providing enzyme reactions that can undergo repeated interrogation or continuous use. The scalability of

the process is an obvious advantage, allowing for large-scale systems applicable to continuous large-scale synthesis, and also to small-scale microfluidic systems that are more suited to preliminary screening. In addition, the IMER process has been shown to be adaptable to a mix and match design in which individual IMER units can be rearranged to change the series of catalytic events, and hence, the product. Coimmobilization of enzymes either in a combined system, or individually as single sequential units will allow for development of complex catalytic sequences. Biologically-derived silica (biosilica) provides a feasible and versatile alternative method for forming a silica matrix that acts as an excellent scaffold for enzyme encapsulation. The biosilica reaction is rapid and simple with high loading capacities and high mechanical stability that has been used in applications such as screening enzyme-drug interactions and for the synthesis of novel chemicals and antibiotic intermediates. The application of IMER technology will doubtless continue, as enzyme immobilization techniques and support matrices continue to be developed.

Acknowledgements

Portions of the research presented herein represent recent research within the Microbiology and Applied Biochemistry program at the Air Force Research Laboratory. This work has been supported by several funding sources including Air Force Office of Scientific Research, Joint Services Technology Office and Air Force Research Laboratory, Material Science Directorate. HRL is an employee of Universal Technology Corporation, 1270 N. Fairfield Road, Dayton, OH.

Table 1. Comparison of IMER preparation methods utilizing cholinesterase enzymes

*The monolith columns included in this table are not silica based but are included for reference and comparison.

†Stability is cited as activity remaining following the time period given

[a]Potentiometric detection, [b]Conductimetric detection, (-) not applicable or not reported, (ID) internal diameter, acetylcholinesterase (AChE), butyrylcholinesterase (BuChE)

	Monolith disks*		**Packed Silica**	**Biosilica**	**Epoxide-Silica**	**Silica-gel**
Enzyme	Human AChE	Human AChE	Human AChE	Equine BuChE	Human AChE	Electric-eel AChE
Column matrix	EDA-CIM disk	Epoxy-CIM disk	Silica gel, (40 μm, 300Å)	Biosilica coated agarose beads	Epoxide silica gel (5 μm, 200Å)	Silica gel (40-63 μm, 60Å)
Dimensions	3x12 mm	3x12 mm	3x35 mm	25x20 mm	50x4.6 mm (ID)	3x0.31 cm
Immobilization	*In situ*	*In situ*	Batch	*In situ*	*In situ*	Batch
Immobilization efficiency	3%	3%	29%	~100%	25%	-
Stability†	<30% (2 mos.)	~80% (2 mos.)	~80% (2 mos.)	100% (15 h)	70% (4 mos.)	Reused 2 – 7 times
Flow Rate (ml/min)	0.2 - 1.4	0.2 - 1.4	0.2 - 1.4	0.5 - 3.0	0.1	0.25 - 1.0
Analysis time (min)	5	5	5	10	20	35–45[a] 31–37[b]
Bed volume (mL)	0.34	0.34	0.06	5.0	-	-
Immobilized enzyme (U)	0.18±0.01	0.22±0.01	4.35±0.01	~160	63	150[a], 200[b]
Reference	[73]	[[73]	[73]	[35]	[74]	[75]

Figure 1. **Use of silica-immobilized enzyme reactors**

Source: Scopus keyword search for *silica immobilized enzyme reactors* in title, abstract and keywords; categorized by year (bar chart) and subject area (inset pie chart).

Figure 2. **Preparation of IMERs using biologically-synthesized silica**

A mixture of a silica-precipitating peptide and hydrolyzed tetramethylorthosilicate rapidly forms silica in aqueous solution (a), producing a network of silica nanospheres of ~500 nm (b). The particles can be attached to large agarose beads (c) by metal affinity binding; by adding six histidine residues to the peptide (d). *Figure adapted from Journal of Chromatography B, 2006, 843(2), 310-316.*[35]

Figure 3. **Screening of a cholinesterase inhibitor on a BuChE-IMER**

Chromatograms showing subsequent injections to a BuChE-IMER of butyrylthiocholine at saturating substrate concentration (a), with 1mM tacrine inhibitor (b) and again with butyrylthiocholine at saturating substrate concentration (c)

Figure 4. **Pathway for dopamine metabolism**

Dopamine β-hydroxylase (DBH), Phenylethanolamine *N*-methyltransferase (PNMT)

Figure 5. **Silica-immobilized nitroreductase IMER**

Schematic of the IMER containing silica-encapsulated nitroreductase inside a stainless steel microreactor (specifications shown) and operational stability of the resulting column for the conversion of nitrobenzene (100 μM) at 5 μl/min. *Figures reprinted with permission from Biomacromolecules, 2006, 7, 2631-2636.* [87] *Copyright 2006 American Chemical Society.*

Figure 6. **Enzyme catalyzed conversion of nitroarenes**
Hydroxylaminobenzene mutase (HABM), Soybean peroxidase (SBPO)

Figure 1.

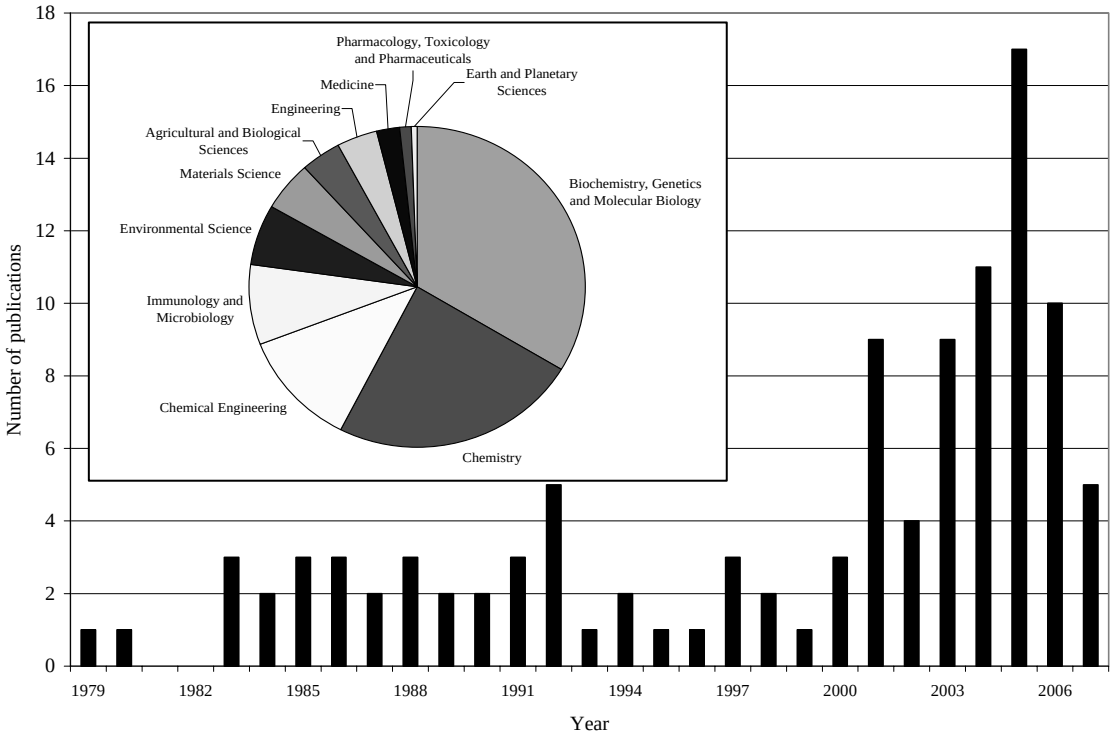

Number of publications
Year
0
2
4
6
8
10
12
14
16
18
1979
1982
1985
1988
1991
1994
1997
2000
2003
2006
Pharmacology, Toxicology and Pharmaceuticals
Earth and Planetary Sciences
Medicine
Engineering
Agricultural and Biological Sciences
Materials Science
Environmental Science
Immunology and Microbiology
Chemical Engineering
Chemistry
Biochemistry, Genetics and Molecular Biology

Figure 2.

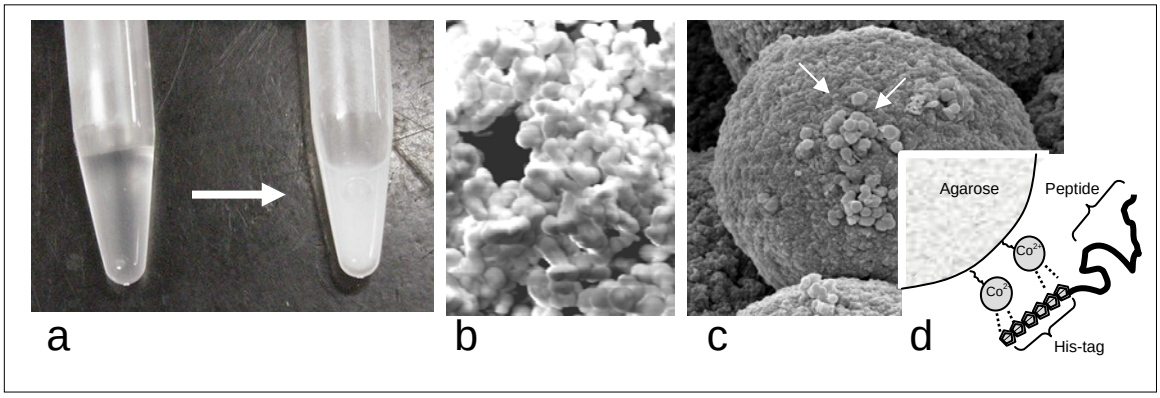


Figure 3.

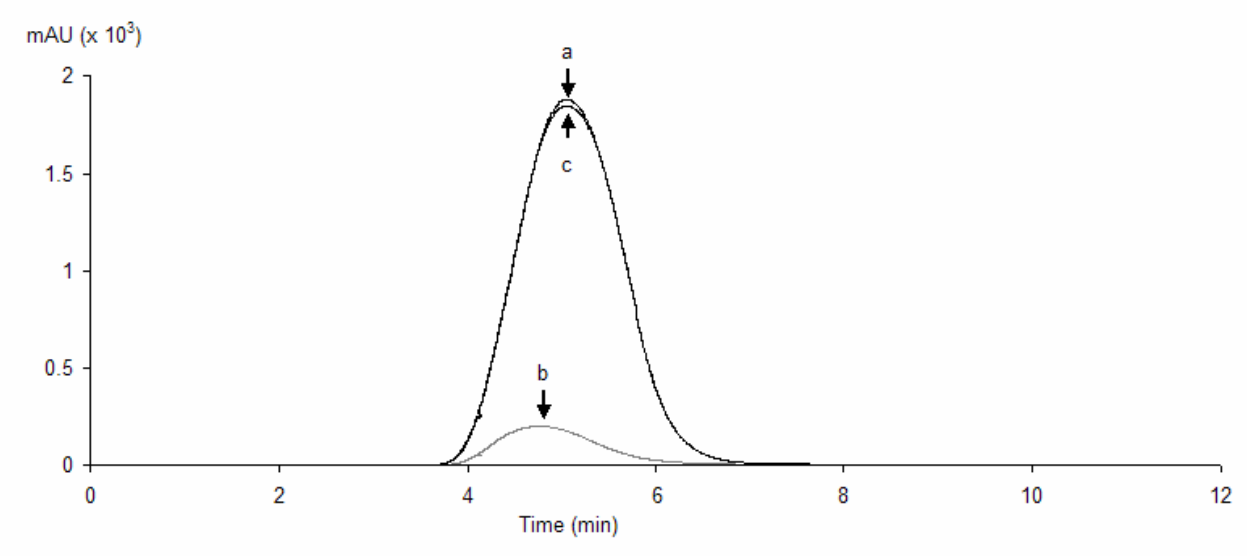
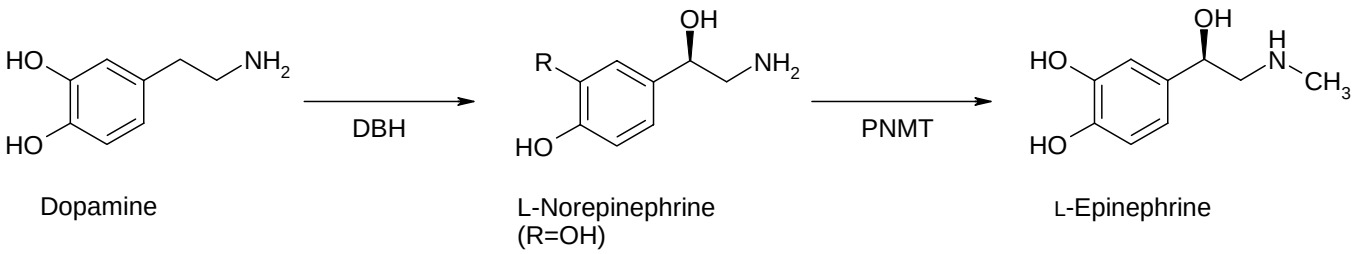


Figure 4.



Dopamine

L-Norepinephrine
(R=OH)

L-Epinephrine

Figure 5.

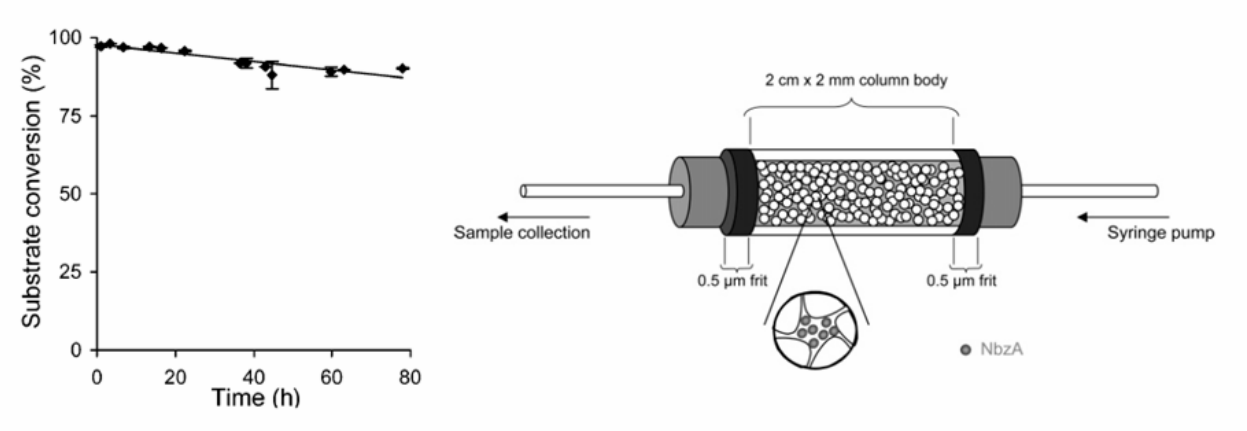
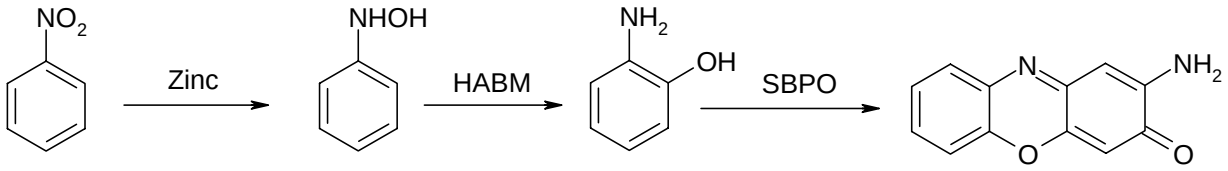
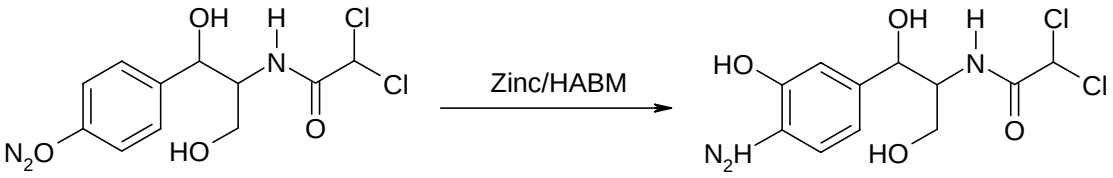

Substrate conversion (%)
100
75
50
25
0
0
20
40
60
80
Time (h)
2 cm x 2 mm column body
Sample collection
Syringe pump
0.5 µm frit
0.5 µm frit
NbzA

Figure 6.



Nitrobenzene Hydroxylaminobenzene *o*-aminophenol 2-aminophenoxazin-3-one



Chloramphenicol

References

1. Liang, J. F.; Li, Y. T.; Yang, V. C. Biomedical application of immobilized enzymes. J. Pharm. Sci. 2000, 89, 979-990.
2. Cao, L. Immobilised enzymes: science or art? Curr. Opin. Chem. Biol. 2005, 9, 217-226.
3. Katchalski-Katzir, E. Immobilized enzymes-learning from past successes and failures. Trends Biotechnol. 1993, 11, 471-478.
4. Bornscheuer, U. T. Immobilizing enzymes: how to create more suitable biocatalysts. Angew. Chem. Int. Ed. 2003, 42, 3336-3337.
5. Sheldon, R. Enzyme Immobilization: The Quest for Optimum Preformance. Adv. Synth. Catal. 2007, 349, 1289-1307.
6. Krenkova, J.; Bilkova, Z.; Foret, F. Characterization of a monolithic immobilized trypsin microreactor with on-line coupling to ESI-MS. J. Sep. Sci. 2005, 28, 1675-1684.
7. Temporini, C.; Perani, E.; Mancini, F.; Bartolini, M.; Calleri, E.; Lubda, D.; Felix, G.; Andrisano, V.; Massolini, G. Optimization of a trypsin-bioreactor coupled with high-performance liquid chromatography-electrospray ionization tandem mass spectrometry for quality control of biotechnological drugs. J. Chromatogr. A 2006, 1120, 121-131.
8. Urban, P. L.; Goodall, D. M.; Bruce, N. C. Enzymatic microreactors in chemical analysis and kinetic studies. Biotechnol. Adv. 2006, 24, 42-57.
9. Massolini, G.; Calleri, E. Immobilized trypsin systems coupled on-line to separation methods: recent developments and analytical applications. J. Sep. Sci. 2005, 28, 7-21.
10. Kato, M.; Inuzuka, K.; Sakai-Kato, K.; Toyo'oka, T. Monolithic bioreactor immobilizing trypsin for high-throughput analysis. Anal. Chem. 2005, 77, 1813-1818.
11. Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microbial Technol. 2007, 40, 1451-1463.
12. Mozahev, V. V.; Melik-Nubarov, N. S.; Sergeeva, M. V.; Sikrnis, V.; Martinek, K. Strategy for stabilizing enzymes. I. Increasing stability of enzymes via their multipoint interaction with a support. Biocatalysis 1990, 3, 179-187.
13. Klibanov, A. M. Stabilization of enzymes against thermal inactivation. Adv. App. Micro. 1983, 29, 1-28.
14. Palomo, J. M.; Fernandez-Lorente, G.; Mateo, C.; Ortiz, C.; Fernandez-Lafuente, R.; Guisan, J. M. Modulation of the enantioselectivity of lipases via controlled immobilization and medium engineering: hydrolytic resolution of mandelic acid esters. Enzyme Microbial Technol. 2002, 31, 775-783.
15. Fernandez-Lafuente, R.; Armisen, P.; Sabuquillo, P.; Fernandez-Lorente, G.; M. Guisan, J. Immobilization of lipases by selective adsorption on hydrophobic supports. Chem. Phys. Lipids 1998, 93, 185-197.
16. Petkar, M.; Lali, A.; Caimi, P.; Daminati, M. Immobilization of lipases for non-aqueous synthesis. J. Mol. Catal. B: Enzymatic 2006, 39, 83-90.
17. Avnir, D.; Lev, O.; Livage, J. Recent bio-applications of sol-gel materials. J. Mat. Chem. 2006, 16, 1013-1030.
18. Gill, I.; Ballesteros, A. Bioencapsulation within synthetic polymers (Part 1): sol-gel encapsulated biologicals. Trends Biotechnol. 2000, 18, 282-296.

19. Besanger, T. R.; Hodgson, R. J.; Green, J. R. A.; Brennan, J. D. Immobilized enzyme reactor chromatography: Optimization of protein retention and enzyme activity in monolithic silica stationary phases. Anal. Chimica Acta 2006, 564, 106-115.
20. Shimizu, K.; Cha, J.; Stucky, G. D.; Morse, D. E. Silicatein alpha: cathepsin L-like protein in sponge biosilica. Proc. Nat. Acad. Sci. 1998, 95, 6234-6238.
21. Cha, J. N.; Shimizu, K.; Zhou, Y.; Christiansen, S. C.; Chmelka, B. F.; Stucky, G. D.; Morse, D. E. Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones in vitro. Proc. Nat. Acad. Sci. 1999, 96, 361-365.
22. Kroger, N.; Deutzmann, R.; Sumper, M. Polycationic peptides from diatom biosilica that direct silica nanosphere formation. Science 1999, 286, 1129-1132.
23. Luckarift, H. R.; Spain, J. C.; Naik, R. R.; Stone, M. O. Enzyme immobilization in a biomimetic silica support. Nat. Biotechnol. 2004, 22, 211-213.
24. Naik, R. R.; Tomczak, M. M.; Luckarift, H. R.; Spain, J. C.; Stone, M. O. Entrapment of enzymes and nanoparticles using biomimetically synthesized silica. Chem. Commun. (Camb) 2004, 1684-1685.
25. Burns, K. L.; May, S. W. Separation methods applicable to the evaluation of enzyme-inhibitor and enzyme-substrate interactions. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2003, 797, 175-190.
26. Emneus, J.; Marko-Varga, G. Biospecific detection in liquid chromatography. J. Chromatogr. A. 1995, 703, 191-243.
27. Ono, M.; Idei, N.; Nakajima, T.; Itoh, Y.; Kawakami, N.; Shimada, K.; Yamato, S. Simultaneous determination of riboflavin phosphate and other ingredients in a multivitamin pharmaceutical preparation by on-line automated LC coupled with pre-column immobilized enzyme reactor. J. Pharm. Biomed. Anal. 2002, 29, 325-334.
28. Yamato, S.; Kawakami, N.; Shimada, K.; Ono, M.; Idei, N.; Itoh, Y. On-line automated high-performance liquid chromatographic determination of total riboflavin phosphates using immobilized acid phosphatase as a pre-column reactor. J. Chromatogr. A 2000, 896, 171-181.
29. Wada, M.; Inoue, K.; Thara, A.; Kishikawa, N.; Nakashima, K.; Kuroda, N. Determination of organic peroxides by liquid chromatography with on-line post-column ultraviolet irradiation and peroxyoxalate chemiluminescence detection. J. Chromatogr. A 2003, 987, 189-195.
30. Marko-Varga, G.; Dominguez, E.; Hahn-Hagerdal, B.; Gorton, L. Bioselective detection in liquid chromatography by the use of immobilized enzymes. J. Pharm. Biomed. Anal. 1990, 8, 817-823.
31. Jansen, H.; Brinkman, U. A.; Frei, R. W. Stereoselective determination of L-amino acids using column liquid chromatography with an enzymatic solid-phase reactor and chemiluminescence detection. J.Chromatogr. 1988, 440, 217-223.
32. Werkhoven-Goewie, C. E.; de Ruiter, C.; Brinkman, U. A.; Frei, R. W.; de Jong, G. J.; Little, C. J.; Stahel, O. Automated determination of drugs in blood samples after enzymatic hydrolysis using precolumn switching and post-column reaction detection. J. Chromatogr. 1983, 255, 79-90.
33. Girelli, A. M.; Mattei, E. Application of immobilized enzyme reactor in on-line high performance liquid chromatography: a review. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2005, 819, 3-16.

34. Kerby, M. B.; Legge, R. S.; Tripathi, A. Measurements of kinetic parameters in a microfluidic reactor. Anal. Chem. 2006, 78, 8273-8280.
35. Luckarift, H. R.; Johnson, G. R.; Spain, J. C. Silica-immobilized enzyme reactors; application to cholinesterase-inhibition studies. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2006, 843, 310-316.
36. Andrisano, V.; Bartolini, M.; Gotti, R.; Cavrini, V.; Felix, G. Determination of inhibitors' potency (IC50) by a direct high-performance liquid chromatographic method on an immobilised acetylcholinesterase column. J. Chromatogr. B. Biomed. Sci. Appl. 2001, 753, 375-383.
37. Jadaud, P.; Wainer, I. W. The stereochemical resolution of the enantiomers of aspartame on an immobilized alpha-chymotrypsin HPLC chiral stationary phase: the effect of mobile-phase composition and enzyme activity. Chirality 1990, 2, 32-37.
38. Gottschalk, I.; Lagerquist, C.; Zuo, S. S.; Lundqvist, A.; Lundahl, P. Immobilized-biomembrane affinity chromatography for binding studies of membrane proteins. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2002, 768, 31-40.
39. Lundqvist, A.; Lundahl, P. Advantages of quantitative affinity chromatography for the analysis of solute interaction with membrane proteins. J. Biochem. Biophys. Methods 2001, 49, 507-521.
40. Moaddel, R.; Lu, L.; Baynham, M.; Wainer, I. W. Immobilized receptor- and transporter-based liquid chromatographic phases for on-line pharmacological and biochemical studies: a mini-review. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2002, 768, 41-53.
41. Bertucci, C.; Bartolini, M.; Gotti, R.; Andrisano, V. Drug affinity to immobilized target bio-polymers by high-performance liquid chromatography and capillary electrophoresis. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2003, 797, 111-129.
42. Kawakami, K.; Sera, Y.; Sakai, S.; Ono, T.; Ijima, H. Development and Characterization of a silica monolith immobilized enzyme micro-bioreactor. Ind. Eng. Chem. Res. 2005, 44, 236-240.
43. Josic, D.; Buchacher, A. Application of monoliths as supports for affinity chromatography and fast enzymatic conversion. J. Biochem. Biophys. Methods 2001, 49, 153-174.
44. Calleri, E.; Massolini, G.; Lubda, D.; Temporini, C.; Loiodice, F. C.; Caccialanza, G. Evaluation of a monolithic epoxy silica support for penicillin G acylase immobilization. J. Chromatogr. A 2004, 1031, 93-100.
45. Ostryanina, N. D.; Vlasov, G. P.; Tennikova, T. B. Multifunctional fractionation of polyclonal antibodies by immunoaffinity high-performance monolithic disk chromatography. J. Chromatogr. A 2002, 949, 163-171.
46. Liu, H.; Carter, G. T.; Tischler, M. Immobilized artificial membrane chromatography with mass spectrometric detection: a rapid method for screening drug-membrane interactions. Rapid Comm. Mass Spec. 2001, 15, 1533-1538.
47. Krause, E.; Dathe, M.; Wieprecht, T.; Bienert, M. Noncovalent immobilized artificial membrane chromatography, an improved method for describing peptide-lipid bilayer interactions. J. Chromatogr. A 1999, 849, 125-133.
48. Luco, J. M.; Salinas, A. P.; Torriero, A. A.; Vazquez, R. N.; Raba, J.; Marchevsky, E. Immobilized artificial membrane chromatography: quantitative structure-retention

relationships of structurally diverse drugs. J. Chem. Info. Computer Sci. 2003, 43, 2129-2136.
49. Masucci, J. A.; Caldwell, G. W.; Foley, J. P. Comparison of the retention behavior of beta-blockers using immobilized artificial membrane chromatography and lysophospholipid micellar electrokinetic chromatography. J. Chromatogr. A 1998, 810, 95-103.
50. Pehourcq, F.; Jarry, C.; Bannwarth, B. Potential of immobilized artificial membrane chromatography for lipophilicity determination of arylpropionic acid non-steroidal anti-inflammatory drugs. J. Pharm. Biomed. Anal. 2003, 33, 137-144.
51. Pidgeon, C.; Stevens, J.; Otto, S.; Jefcoate, C.; Marcus, C. Immobilized artificial membrane chromatography: rapid purification of functional membrane proteins. Anal. Biochem. 1991, 194, 163-173.
52. Pidgeon, C.; Venkataram, U. V. Immobilized artificial membrane chromatography: supports composed of membrane lipids. Anal. Biochem. 1989, 176, 36-47.
53. Stewart, B. H.; Chan, O. H. Use of immobilized artificial membrane chromatography for drug transport applications. J. Pharm. Sci. 1998, 87, 1471-1478.
54. Yang, C. Y.; Cai, S. J.; Liu, H.; Pidgeon, C. Immobilized artificial membranes - screens for drug membrane interactions. Adv. Drug Delivery Rev. 1996, 23, 229-256.
55. Cloix, J. F.; Wainer, I. W. Development of an immobilized brain glutamine synthetase liquid chromatographic stationary phase for on-line biochemical studies. J. Chromatogr. A. 2001, 913, 133-140.
56. Luckarift, H. R.; Nadeau, L. J.; Spain, J. C. Continuous synthesis of aminophenols from nitroaromatic compounds by combination of metal and biocatalyst. Chem. Commun. (Camb) 2005, 383-384.
57. Luckarift, H. R.; Balasubramanian, S.; Paliwal, S.; Johnson, G. R.; Simonian, A. L. Enzyme-encapsulated silica monolayers for rapid functionalization of a gold surface. Coll. Surf. B Biointerfaces 2007, 58, 28-33.
58. Luckarift, H.; Ku, B.; Dordick, J.; Spain, J. Silica-Immobilized Enzymes for multi-step synthesis in microfluidic devices. Biotech. Bioeng. 2007, 98, 701-705.
59. Luckarift, H.; Greenwald, R.; Bergin, M.; Spain, J.; Johnson, G. Biosensor system for continuous monitoring of organophosphate aerosols. Biosens. Bioelectron. 2007. *In Press.*
60. Yao, L. X.; Wu, Z. C.; Ji, Z. L.; Chen, Y. Z.; Chen, X. Internet resources related to drug action and human response: a review. Appl. Bioinformatics 2006, 5, 131-139.
61. Dannhardt, G.; Kiefer, W. Cyclooxygenase inhibitors-current status and future prospects. Eur. J. Med. Chem. 2001, 36, 109-126.
62. Asberg, M.; Eriksson, B.; Martensson, B.; Traskman-Bendz, L.; Wagner, A. Therapeutic effects of serotonin uptake inhibitors in depression. J. Clin. Psychiatry 1986, 47 Suppl, 23-35.
63. Lemberger, L.; Fuller, R. W.; Zerbe, R. L. Use of specific serotonin uptake inhibitors as antidepressants. Clin. Neuropharmacol. 1985, 8, 299-317.
64. Youdim, M. B.; Edmondson, D.; Tipton, K. F. The therapeutic potential of monoamine oxidase inhibitors. Nat. Rev. Neurosci. 2006, 7, 295-309.
65. Liston, D. R.; Nielsen, J. A.; Villalobos, A.; Chapin, D.; Jones, S. B.; Hubbard, S. T.; Shalaby, I. A.; Ramirez, A.; Nason, D.; White, W. F. Pharmacology of selective

acetylcholinesterase inhibitors: implications for use in Alzheimer's disease. Eur. J. Pharmacol. 2004, 486, 9-17.
66. Holden, M.; Kelly, C. Use of cholinesterase inhibitors in dementia. Adv. Psychiatric Treatment 2002, 8, 89-96.
67. Darvesh, S.; Walsh, R.; Kumar, R.; Caines, A.; Roberts, S.; Magee, D.; Rockwood, K.; Martin, E. Inhibition of human cholinesterases by drugs used to treat Alzheimer disease. Alzheimer Dis. Assoc. Disord. 2003, 17, 117-126.
68. Giacobini, E. Cholinesterase inhibitors: new roles and therapeutic alternatives. Pharmacol. Res. 2004, 50, 433-440.
69. Birks, J. Cholinesterase inhibitors for Alzheimer's disease. Cochrane Database Syst Rev 2006, CD005593.
70. Hitzeman, N. Cholinesterase inhibitors for Alzheimer's disease. Am. Fam. Physician 2006, 74, 747-749.
71. Bajgar, J. Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. Adv Clin Chem 2004, 38, 151-216.
72. Wiener, S. W.; Hoffman, R. S. Nerve agents: a comprehensive review. J Intensive Care Med 2004, 19, 22-37.
73. Bartolini, M.; Cavrini, V.; Andrisano, V. Choosing the right chromatographic support in making a new acetylcholinesterase-micro-immobilised enzyme reactor for drug discovery. J. Chromatogr. A 2005, 1065, 135-144.
74. Bartolini, M.; Cavrini, V.; Andrisano, V. Monolithic micro-immobilized-enzyme reactor with human recombinant acetylcholinesterase for on-line inhibition studies. J. Chromatogr. A 2004, 1031, 27-34.
75. Suwansa-ard, S.; Kanatharana, P.; Asawatreratanakul, P.; Limsakul, C.; Wongkittisuksa, B.; Thavarungkul, P. Semi disposable reactor biosensors for detecting carbamate pesticides in water. Biosens. Bioelectron. 2005, 21, 445-454.
76. Dong, Y.; Wang, L.; Shangguan, D.; Zhao, R.; Liu, G. Improved method for the routine determination of acetylcholine and choline in brain microdialysate using a horseradish peroxidase column as the immobilized enzyme reactor. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2003, 788, 193-198.
77. Kim, H. S.; Wainer, I. W. The covalent immobilization of microsomal uridine diphospho-glucuronosyltransferase (UDPGT): initial synthesis and characterization of an UDPGT immobilized enzyme reactor for the on-line study of glucuronidation. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2005, 823, 158-166.
78. Sakai-Kato, K.; Kato, M.; Toyo'oka, T. On-line drug-metabolism system using microsomes encapsulated in a capillary by the sol-gel method and integrated into capillary electrophoresis. Anal Biochem 2002, 308, 278-284.
79. Calleri, E.; Marrubini, G.; Massolini, G.; Lubda, D.; de Fazio, S. S.; Furlanetto, S.; Wainer, I. W.; Manzo, L.; Caccialanza, G. Development of a chromatographic bioreactor based on immobilized beta-glucuronidase on monolithic support for the determination of dextromethorphan and dextrorphan in human urine. J. Pharm. Biomed. Anal. 2004, 35, 1179-1189.
80. Tsunoda, M. Recent advances in methods for the analysis of catecholamines and their metabolites. Anal. Bioanal. Chem. 2006, 386, 506-514.

81. Markoglou, N.; Wainer, I. W. Biosynthesis in an on-line immobilized-enzyme reactor containing phenylethanolamine N-methyltransferase in single-enzyme and coupled-enzyme formats. J. Chromatogr. A 2002, 948, 249-256.
82. Markoglou, N.; Wainer, I. W. Synthesis and characterization of immobilized dopamine beta-hydroxylase in membrane-bound and solubilized formats. J. Biochem. Biophys. Methods 2001, 48, 61-75.
83. Markoglou, N.; Wainer, I. W. Synthesis and characterization of an immobilized phenylethanolamine N-methyltransferase liquid chromatographic stationary phase. Anal. Biochem. 2001, 288, 83-88.
84. Markoglou, N.; Hsuesh, R.; Wainer, I. W. Immobilized enzyme reactors based upon the flavoenzymes monoamine oxidase A and B. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2004, 804, 295-302.
85. Markoglou, N.; Wainer, I. W. On-line synthesis utilizing immobilized enzyme reactors based upon immobilized dopamine beta-hydroxylase. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2002, 766, 145-151.
86. Goto, Y.; Grace, A. A. The Dopamine System and the Pathophysiology of Schizophrenia: A Basic Science Perspective. Int. Rev. Neurobiol. 2007, 78C, 41-68.
87. Berne, C.; Betancor, L.; Luckarift, H. R.; Spain, J. C. Application of a microfluidic reactor for screening cancer prodrug activation using silica-immobilized nitrobenzene nitroreductase. Biomacromol. 2006, 7, 2631-2636.
88. Opperdoes, F. R. Topogenesis of glycolytic enzymes in Trypanosoma brucei. Biochem. Soc. Symp. 1987, 53, 123-129.
89. Bartolini, M.; Andrisano, V.; Wainer, I. W. Development and characterization of an immobilized enzyme reactor based on glyceraldehyde-3-phosphate dehydrogenase for on-line enzymatic studies. J. Chromatogr. A 2003, 987, 331-340.
90. Straathof, A. J.; Panke, S.; Schmid, A. The production of fine chemicals by biotransformations. Curr. Opin. Biotechnol. 2002, 13, 548-556.
91. Bhat, M. K. Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 2000, 18, 355-383.
92. Panke, S.; Held, M.; Wubbolts, M. Trends and innovations in industrial biocatalysis for the production of fine chemicals. Curr. Opin. Biotechnol. 2004, 15, 272-279.
93. Michels, P. C.; Khmelnitsky, Y. L.; Dordick, J. S.; Clark, D. S. Combinatorial biocatalysis: a natural approach to drug discovery. Trends Biotechnol. 1998, 16, 210-215.
94. Faber, K.; Franssen, M. C. Prospects for the increased application of biocatalysts in organic transformations. Trends Biotechnol. 1993, 11, 461-470.
95. Dordick, J. S.; Khmelnitsky, Y. L.; Sergeeva, M. V. The evolution of biotransformation technologies. Curr. Opin. Microbiol. 1998, 1, 311-318.
96. Dordick, J. S. Biocatalysis in nonaqueous media. Patents and literature. Appl. Biochem. Biotech. 1988, 19, 103-112.
97. Pfromm, P. H.; Rezac, M. E.; Wurges, K.; Czermak, P. fumed silica activated subtilisin carlsberg in hexane in a packed-bed reactor. AIChE Journal 2007, 53, 237-242.
98. Kallenberg, A. I.; Van Rantwijk, F.; Sheldon, R. A. Immobilization of Penicillin G acylase: The key to optimum performance. Adv. Synth. Catal. 2005, 347, 905-926.
99. Giordano, R. C.; Ribeiro, M. P.; Giordano, R. L. Kinetics of beta-lactam antibiotics synthesis by penicillin G acylase (PGA) from the viewpoint of the industrial enzymatic reactor optimization. Biotechnol. Adv. 2006, 24, 27-41.

100. Calleri, E.; Temporini, C.; Massolini, G.; Caccialanza, G. Penicillin G acylase-based stationary phases: analytical applications. J. Pharm. Biomed. Anal. 2004, 35, 243-258.
101. Massolini, G.; Calleri, E.; De Lorenzi, E.; Pregnolato, M.; Terreni, M.; Felix, G.; Gandini, C. Immobilized penicillin G acylase as reactor and chiral selector in liquid chromatography. J. Chromatogr. A 2001, 921, 147-160.
102. Massolini, G.; Calleri, E.; Lavecchia, A.; Loiodice, F.; Lubda, D.; Temporini, C.; Fracchiolla, G.; Tortorella, P.; Novellino, E.; Caccialanza, G. Enantioselective hydrolysis of some 2-aryloxyalkanoic acid methyl esters and isosteric analogues using a penicillin G acylase-based HPLC monolithic silica column. Anal. Chem. 2003, 75, 535-542.
103. Pogori, N.; Xu, Y.; Cheikhyousseff, A. Potential aspects of lipases obtained from Rhizopus fungi. Research Journal of Microbiology 2007, 2, 101-116.
104. Ghanem, A.; Aboul-Enein, H. Y. Application of lipases in kinetic resolution of racemates. Chirality 2005, 17, 1-15.
105. Ghanem, A. Trends in lipase-catalyzed asymmetric access to enantiomerically pure/enriched compounds. Tetrahedron 2007, 63, 1721-1754.
106. Dominguez de Maria, P.; Sinisterra, J. V.; Tsai, S.-W.; Alcantara, A. R. Carica papaya lipase (CPL): An emerging and versatile biocatalyst. Biotechnology Advances 2006, 24, 493-499.
107. Dominguez de Maria, P.; Carboni-Oerlemans, C.; Tuin, B.; Bargeman, G.; Van Der Meer, A.; Van Gemert, R. Biotechnological applications of Candida antarctica lipase A: State-of-the-art. Journal of Molecular Catalysis B: Enzymatic 2005, 37, 36-46.
108. Villeneuve, P.; Muderhwa, J. M.; Graille, J.; Haas, M. J. Customizing lipases for biocatalysis: A survey of chemical, physical and molecular biological approaches. Journal of Molecular Catalysis - B Enzymatic 2000, 9, 113-148.
109. Calleri, E.; Temporini, C.; Furlanetto, S.; Loiodice, F.; Fracchiolla, G.; Massolini, G. Lipases for biocatalysis: development of a chromatographic bioreactor. J. Pharm. Biomed. Anal. 2003, 32, 715-724.
110. Mullangi, R.; Yao, M.; Srinivas, N. R. Resolution of enantiomers of ketoprofen by HPLC: a review. Biomed Chromatogr 2003, 17, 423-434.
111. Jamali, F.; Brocks, D. R. Clinical pharmacokinetics of ketoprofen and its enantiomers. Clin Pharmacokinet 1990, 19, 197-217.
112. Ujang, Z.; Husain, W. H.; Seng, M. C.; Rashid, A. H. A. The kinetic resolution of 2-(4-chlorophenoxy) propionic acid using Candida rugosa lipase. Proc. Biochem. 2003, 38, 1483-1488.
113. Wedin, G. P.; Neal, J. S.; Everson, G. W.; Krenzelok, E. P. Castor bean poisoning. Am. J. Emerg. Med. 1986, 4, 259-261.
114. Challoner, K. R.; McCarron, M. M. Castor bean intoxication. Ann. Emerg. Med. 1990, 19, 1177-1183.
115. Biosensor. In IUPAC Compendium of Chemical Terminology, 1992, p 148.
116. Fishman, H. A.; Greenwald, D. R.; Zare, R. N. Biosensors in chemical separations. Annu. Rev. Biophys. Biomol. Struct. 1998, 27, 165-198.